

IN THE CLAIMS

This listing of claims replaces all prior versions, and listings, in this application.

1. (currently amended) A process for preparation and purification of recombinant human interferon (hu-IFN) alpha 2b which comprises-of:

- I. cultivating recombinant *Pichia pastoris* containing a hu-IFN alpha 2b gene, [[.]]
- II. culturing said recombinant *Pichia pastoris* in complex/defined salt culture medium to produce hu-IFN alpha 2b protein, and [[.]]
- III. purifying recombinant hu-IFN alpha 2b protein from said culture medium.

2. (currently amended) A process as claimed in claim 1 wherein said human IFN alpha 2b gene comprises (SEQ ID NO:3) of the following sequence:

Nucleotide sequence of recombinant human IFN alpha 2b gene:

SEQ ID 3:

TGT	GAT	CTG	CCT	CAA	AGC	CAG	AGC	CTG	GGT	AGC	AGG	AGG	ACC	TTG	ATG
CTG	CTG	GCG	CAG	ATG	AGG	AGA	ATC	TCT	CTT	TTG	TGG	TGG	TTG	AAG	GAG
AGA	CAT	GAC	TTT	GGA	TTT	CCC	CAG	GAG	GAG	TTT	GGC	AAC	CAG	TTG	CAA
AAG	GCT	GAA	AGC	ATC	CCT	GTG	CTG	CAT	GAG	ATG	ATC	CAG	CAG	ATC	TTG
AAG	CTC	TTG	AGG	ACA	AAG	GAG	TCA	TCT	GCT	GCT	TGG	GAT	GAG	ACC	CTC
CTA	GAC	AAA	TTG	TAG	ACT	GAA	CTG	TAG	CAG	CAG	CTG	AAT	GAC	CTG	GAA
GCC	TGT	GTG	ATA	CAG	GGG	GTG	GGG	GTG	ACA	GAG	ACT	CCC	CTG	ATG	AAG
GAG	GAC	TCC	ATT	CTG	GCT	GTG	AGG	AAA	TAC	TTG	CAA	AGA	ATC	ACT	CTC
TAT	CTG	AAA	GAG	AAG	AAA	TAC	AGC	CCT	TGT	GCC	TGG	GAG	GTT	GTG	AGA
GCA	GAA	ATC	ATG	AGA	TCT	TTT	TCT	TTG	TCA	ACA	AAC	TTG	CAA	GAA	AGT
TTA	AGA	AGT	AAG	GAA	TCA*										

(* = Stop codon.)

3. (currently amended) A process as claimed in Claim 1 wherein said recombinant *Pichia pastoris* containing a hu-IFN alpha 2b gene is cultivated by first isolating and purifying mRNA from human leucocytes, preparing a first strand of DNA from said purified mRNA to obtain said modified hu-IFN alpha 2b gene, amplifying said gene and cloning said amplified modified hu-IFN alpha 2b gene into an expression vector, [[.]] amplifying and isolating said hu-IFN alpha 2b gene from said modified interferon alpha

2b clone, and cloning said hu-IFN alpha 2b gene ~~[[it]]~~ into an expression vector and transforming said expression vector ~~[[it]]~~ into said *Pichia pastoris*.

4. (currently amended) A process as claimed in claim 1 wherein said cloning is carried out ~~[[our]]~~ by RT-PCR methods employing primer pairs having the sequence selected from the group consisting of SEQ ID NOS: SEQ ID 4 & 5; 6 & 7; 8 & 9; 10 & 11; 12 & 13 and 12 & 14.

5. (currently amended) A process as claimed in Claim 3 wherein said *Pichia pastoris* ~~[[host]]~~ is selected from *Pichia pastoris* KM 71, *Picha pastoris* KM 71H, *Pichia pastoris* GS115, *Pichia pastoris* X33 preferably *Pichia pastoris* KM71, *Pichia*

6. (original) A process as claimed in Claim 3 wherein said vector is pPICZ α A.

7. (currently amended) A process as claimed in Claim 6 wherein said hu-IFN alpha 2b gene is cloned in pPICZ α A vector down stream to AOX promoter and alpha mat signal sequence.

8. (currently amended) A process as claimed in Claim 3 wherein a desired construct containing a hu-IFN alpha 2b gene (expression cassette) is integrated at the AOX region ~~in desired site of *Pichia pastroris* genome, at the AOX region.~~

9. (currently amended) A process as claimed in Claim 8 wherein said expression cassette is integrated at the 5' AOX region of ~~[[host]]~~ *Pichia pastoris* selected from *Pichia pastoris* KM 71, *Picha pastoris* KM 71H, *Pichia pastoris* GS115, *Pichia pastoris* X33 preferably *Pichia pastoris* KM71.

10. (currently amended) A process as claimed in Claim 9 wherein said *Pichia pastoris* has His auxotrophic phenotype.

11. (original) A process as claimed in Claim 1 wherein said culture medium is selected from complex media like BGY, BY, BMY, BGYP, YPD, defined salt medium preferably defined salt medium and BMY.

12. (currently amended) A process as claimed in Claim 11 wherein said culture medium comprises one or more ~~[[a]]~~ nitrogen sources selected from the group consisting one or more of ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, bean-cakes, potato extracts, protein hydrolysates, yeast extract, urea and ammonium hydroxide.

13. (currently amended) A process as claimed in Claim 1 wherein said culture medium comprises ~~[[of]]~~ a carbon source such as glycerol, glucose, fructose, methanol and the like, preferably glycerol.

14. (currently amended) A process as claimed in Claim 1 wherein the biomass build up is in a range of ~~from~~ 35 to 100 g/L, preferably 35-50 g/L for complex medium ~~media~~ and 50 to 80 g/L, preferably 50-60 g/L for defined salt medium ~~media~~ based on dry cell weight.

15. (currently amended) A process as claimed in claim 14 wherein said culture medium has:

- (a) pH in the range of 3.0 to 6.0, preferably 6.0 to 6.5 for complex medium ~~media~~ and 3.5 to 4.5 for defined salt medium ~~media~~ preferably 5.8 to 6.2, ~~[[.]]~~
- (b) temperature in the range of 25 to 35°~~[[°]]~~C preferably 28 to 32°C, and ~~[[.]]~~
- (c) dissolved oxygen: 20-80% of saturation, preferably 40-50% of saturation and said culturing is carried out for a duration of 48 to 110 hours, preferably 48 to 72 hours for complex medium ~~media~~ and 90-110 hours for defined salt medium ~~media~~.

16. (previously presented) A process as claimed in Claim 11 wherein the expression of recombinant IFN alpha 2b protein is induced after reaching appropriate biomass buildup

using suitable alcohol such as methanol, ethanol and the like preferably methanol at concentration of 0.1 to 3.0% v/v, preferably 1-1.5% v/v.

17. (original) A process as claimed in Claim 16, wherein the expression of full length recombinant IFN alpha 2b protein is regulated by addition of nitrogen source selected from yeast nitrogen base, yeast nitrogen base without amino acid, yeast hydrolysate, yeast extract, peptone, casamino acid, meat extract, beef extract and like, preferably yeast extract and peptone along with or without propylene glycol.

18. (currently amended) A process as claimed in Claim 1 ~~any preceding claim~~ wherein said recombinant hu-IFN alpha 2b protein is purified to homogeneity by

- (a) ~~separating the cells from the cell culture to obtain~~[[e]] the supernatant which contains ~~expressed~~ recombinant hu-IFN alpha 2b protein; [[.]]
- (b) ~~subjecting~~ said supernatant to [[a]] cation exchange chromatography by
 - (i) ~~binding said expressed~~ recombinant hu-IFN alpha 2b protein on a column packed with either CM SEPHAROSE sepharose FF, SP SEPHAROSE sepharose FF or SEPRAPREP sepraprep S₁
 - (ii) ~~washing~~ said column with a buffer selected from citrate, phosphate, acetate buffer or CIEXI buffer, at a pH 5.0-5.5 to remove unwanted proteins, and [[.]]
 - (iii) ~~eluting~~ Eluting said recombinant hu-IFN alpha 2b protein with CIEXI buffer with pH 4.8-5.4; [[.]]
- (c) ~~subjecting~~ Subjecting the eluent obtained in step (b)(iii) to anion exchange chromatography followed by elution with ALEX II buffer; [[.]]
- (d) ~~subjecting~~ Subjecting the eluent from step (c) to ultrafiltration ~~using Amicon stirred cell with YM-10 membrane of pore size 10,000 Dalton molecular cut off~~ to obtain a concentrated retentate containing recombinant hu-IFN alpha 2b protein;
- (e) ~~subjecting~~ Subjecting said concentrated retentate to gel filtration chromatography using ammonium acetate buffer containing Tween-80 and

EDTA, pH 5.2-5.5, to obtain homogenous species of recombinant hu-IFN alpha 2b protein; and [[.]]

- (f) purifying said recombinant hu-IFN alpha 2b protein obtained in step (e) by repeating steps (a) to (e) in any sequence or order.

19. (currently amended) A pharmaceutical composition comprising ~~of~~ purified interferon alpha 2b prepared and purified according to claim 1, and a pharmaceutically acceptable carrier either in liquid form or in lyophilized form.

20. (currently amended) A pharmaceutically composition as [[a]] claimed in Claim 19 wherein said pharmaceutically acceptable carrier [[salt]] comprises phosphate buffer, glycine, HSA, PEG, ammonium acetate, NaCl, Tween-80, EDTA, Benzyl alcohol and the like in any combination and with desired concentration / amount.

21. (currently amended) A method of treatment using ~~and use of~~ purified interferon alpha 2b prepared and purified according to claim 1 ~~of the present invention in the preparation of medicament~~ for treatment of viral diseases like chronic active Hepatitis B, Chronic active Hepatitis non A-non B, Chronic active Hepatitis delta, Chronic active Hepatitis C; cancer diseases like Chronic myelogenous leukemia, Non-Hodgkin's lymphoma, AIDS related Kaposi's Sarcoma, Renal cell carcinoma, Malignant melanoma, Hairy cell leukemia, Bladder carcinoma, Superficial and noduloulcerative basal cell carcinoma, Condylomata acuminata, Laryngeal papillomatosis, and like.